Investigation on the Toxic & Teratogenic Effects of GRAS Substances on the Developing Chick Embryo **Zinc Sulfate** No Date

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Investigations on the Toxic and Teratogenic Effects of GRAS Substances on the Developing Chick Embryo.[1]

Zinc. Sulfate

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[1]Report of investigations conducted under Contract No. 72-343 with the Food and Drug Administration, PHS, DHEW.

General Protocol:

Ten test substances were supplied by the Food and Drug Administration for testing in the chick embryo. Details on the nature and source of these substances is shown in Table i. All substances were stored at room temperature in the dark until they were used, except that the propyl gallate and phosphated mono- and di-glycerides were kept under refrigeration. Most of the substances were dissolved in a suitable solvent or suspended in a suitable liquid for injection into fertile eggs. In one instance the substance was injected directly without a solvent or carrier. Specific information about solvents, solubility of the substances and problems peculiar to individual substances will be given under specific protocol for each substance tested.

Fertile eggs used in these investigations were from a specific pathogen free flock of Dekalb 161 egg production type chickens fed a breeder ration free of antibiotics or other drugs. Eggs were stored at 55° F and a relative humidity of 80 percent for 0 to 5 days prior to use. Eggs were allowed to reach room temperature, placed on plastic flats and subjected to ultraviolet irradiation for 30 minutes. The top of each egg was cleansed by a cotton swab saturated with 70 percent ethanol, a small hole was drilled over the air cell through the shell and the test substance was injected with the aid of a 0.25 ml. tuberculin syringe fitted with a suitable needle. All equipment and glassware used to handle the test substances or their solutions or suspensions were sterilized by auto claving and every attempt was made to avoid microbiological contamination of the eggs. Following injection the hole in each egg was sealed by a drop of flexible collodion and the eggs were set in or returned to the incubators. Jamesway Model 252 Incubator-Hatchers were used and maintained at 100° F dry bulb temperature and 86° F wet bulb temperature during the first 18 days of incubation. Eggs were turned automatically each 4 hours. Eggs were candled periodically to remove dead embryos and all embryos were examined for stage of development and obvious defects. After 18 days of incubation viable embryos were transferred to hatching baskets and hatching temperature was reduced to 99.5° F dry bulb reading and humidity was increased to a 90° F wet bulb reading. Upon hatching (22nd day) chicks were examined for abnormalities and samples were cleared and alizarin stained to examine them for skeletal defects. Other embryos (50 for each substance studied) were sacrificed and samples of liver, muscle, bursa, brain, eye, spleen, heart, pancreas, lung and kidney were taken and fixed in formalin. Later tissues were embeded in paraffin, cut, stained and mounted for histopathological examination. Each sample was done in duplicate and hence a total of 10,000 tissues were examined for lesions.

Preliminary range finding experiments were conducted to find the doses of the test substances that could be used in constructing dose response curves for toxicity as measured by embryonic mortality. In two cases, the test substance was non-toxic in the largest dose that could be accommodated by injection. Specific dose response experiments using 100 or more eggs per dose and 5 or more doses of the test substance were conducted at a minimum of 3 time intervals to obtain the toxicity data reported. Solvent or sham injected controls and untreated control groups of eggs were used with each experiment. In some cases, extra trials were conducted to provide embryos for examination at critical doses of the test substances in order to further evaluate teratogenic response and obtain additional data on the nature of embryonic defects.

Data obtained from the experiments (except that from the range finding studies) was transferred to data sheets provided (FDH form 2572, 2572a and 2572b) and submitted to FDA for statistical analysis. Nine types of data summaries including 2 statistical treatments of the data were provided by FDA on the data submitted. The results presented and interpretations made are largely based on these data summaries.

Table i

FDA Project Test Substances

Test Substance and Identification		Compound No.
1. Lactose, Edible Formost Dairies, Inc. Appleton, Wisc.		000063423
2. Propyl Gallate Lot 337		000121799
3. Sodium Ascorbate, U.S.P. FCC Lot No. 965102 Hoffmann-LaRoche Inc., Nutley, N. J. FDA 3167 73(C)		000134032
4. Sodium Erythorbate F.C.C. Lot No. 834072 FDA 3167 73(C) Hoffmann-LaRoche, Nutley, N. J.		977052064
5. Oil Nutmeg NF, East Indian Fritzsche Dodge & Olcott, Inc. 71-28 New York, N. Y.		MX 8008455
6. Zinc Sulfate - Rayon Lot # 2132R1 Virginia Chemicals, Inc. Portsmouth, Va.	Anhyd. Monohyd.	007733020 007446197
7. Stannous Chloride, AR $2H_2O$ Mallinckrodt Chemical Works St. Louis, Mo.		007772998
8. Talc USP #141, Whittaker, Clark and Daniels, Inc.		010101390
9. Carob Bean Gum FDA 71-14		PM 9000402
10. Phosphated Mono- and Di-Glycerides Lot No. 126 Witco Chemical Organics Division New York, N. Y. EMCOL D70-30C		977051323

General Discussion and Comparisons:

A comparison of the relative toxicity of the ten compounds tested is shown in Table ii. When toxicity is evaluated by the air cell route of injection at 96 hrs. of incubation, which was the most sensitive for most of the substances tested, it may be seen that the test substances can be divided into 3 categories of toxicity. Substances highly toxic are zinc sulfate, propyl gallate and carob bean gum. Moderate toxicity was encountered with sodium ascorbate, sodium erythorbate, oil of nutmeg and stannous chloride. Those substances of low toxicity were lactose, talc and phosphated mono- and di-glyceride.

Most of the substances tested produced general embryo toxic response as ascites and/or edema except for lactose and talc at the doses tested. Some specific structural defects were noted and seemed to be related to certain substances as shown in Table ii.

Table ii

Comparison of Ten Substances Tested
for Toxicity and Teratology

Substance Tested	LC_{50} via air cell at 96 hrs.	Specific Abnormalities Noted
Lactose	very large	none
Propyl Gallate	13 mgs./kg.	Ascites, edema, celosomia.
Sodium Ascorbate	100 mgs./kg.	Ascites, edema, celosomia, liver histopathology, head defects.
Sodium Erythorbate	84 mgs./kg.	Ascites, liver histopathology.
Oil of Nutmeg	240 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Zinc Sulfate	4 mgs./kg.	Ascites, edema, celosomia, dwarfism.
Stannous Chloride	120 mgs./kg.	Ascites, edema, celosomia.
Talc	>200 mgs./kg.	none
Carob Bean Gum	23 mgs./kg.	Anophthalmia, phocomelia, micromelia, torticollis, celosomia.
Phosphated Mono- and Di-Glycerides	>3000 mgs./kg.	Ascites, anophthalmia, brachygnathia.

VI. ZINC SULFATE (RAYON)

Specific Protocol:

Since the product tested was not chemically pure $ZnSO_4$ but a mixture of $ZnSO_4$ and some $Zn(OH)_2$, the product was not easily water soluble as would have been expected. Hence the pH of the product in water was adjusted using H_2SO_4 to give a pH of 4.5 in the resultant solution. Since most of the H_2SO_4 added was used to neutralize the $Zn(OH)_2$ in the test product, the solvent control injections were made using a water solution of H_2SO_4 at a pH of 4.5 rather than using the same amount of total acid used in solublizing the product. Solutions were sterilized by autoclaving because of their stability. Five to 8 dose levels of zinc sulfate (rayon) were tested both at 0 and 96 hrs. of incubation and via both air cell and yolk routes of administration.

Results:

The data for zinc sulfate-rayon is presented in Tables 21-24. Since the computer failed to recognize the solvent control as appropriate for comparison all statistical inferences for this compound are made by comparison to the lowest test dose for the compound under each condition of administration. Under all conditions of administration, percent mortality was increased by the highest test dose used and the regression of dose on mortality was highly significant in all cases. The sensitivity of the embryo to zinc sulfate-rayon was much greater when given at 96 hrs. via the air cell than it was under the other conditions of administration. Significant or highly significant increases in percent abnormal chicks hatched were found at some dose level and under all conditions of compound administration. Percent H-S-V-L abnormalities were increased significantly by yolk administration at both time intervals but not by air cell administration. Significant increases in ascites, edema, celoscamia and dwarfism were noted as teratogenic findings under all conditions. The increased incidence of celoscmia was responsible for the larger H-S-V-L abnormalities found with yolk administration of the test compound.

Discussion:

Zinc sulfate (Rayon) clearly produces an embryo toxic response that is closely related to the dose administered. Both percent mortality and percent abnormal chicks hatched were increased by the substance. While most other compounds tested in this study were more toxic via air cell administration at 96 hrs. of development than by other methods, the toxicity of zinc sulfate-rayon was greatly enhanced by the 96 hrs. administration via the air cell. The LC_{50} at 96 hrs. via the air cell was about 4 mgs./kg.

O LAB 4 ZINC SULFATE RAYON (MG/KG) IN .03 M H2SO4/A/000 ONE OR MORE ABNORMALITIES

* LAB 4 ZINC SULFATE RAYON (MG/KG) IN .03 M H2SO4/A/000 MORTALITY PCT

Lab 4

⊖ LAB 4 ZINC SULFATE RAYON (MG/KG) IN .02 M H2SO4/A/096 ONE OR MORE ABNORMALITIES
✱ LAB 4 ZINC SULFATE RAYON (MG/KG) IN .02 M H2SO4/A/096 MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
MG/KG
0.00
4.00
8.00
12.00
16.00
20.00

Lab 4

O LAB 4 ZINC SULFATE RAYON (MG/KG) IN .03 M H2SO4/Y/000 ONE OR MORE ABNORMALITIES
* LAB 4 ZINC SULFATE RAYON (MG/KG) IN .03 M H2SO4/Y/000 MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
MG/KG
0.00
80.00
160.00
240.00
320.00
400.00

Lab 4

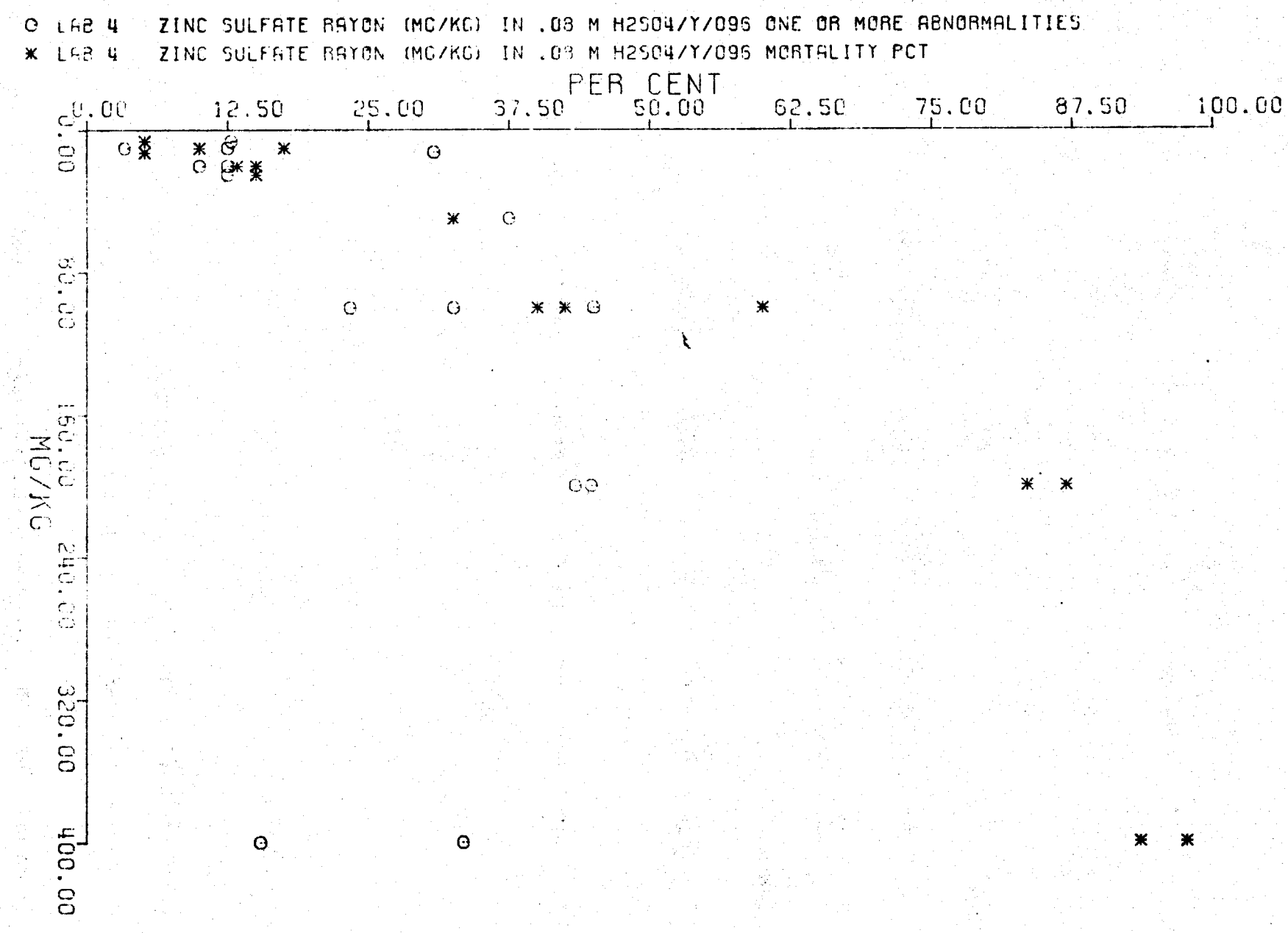
O LAB 4 ZINC SULFATE RAYON (MG/KG) IN .08 M H2SO4/Y/096 ONE OR MORE ABNORMALITIES
* LAB 4 ZINC SULFATE RAYON (MG/KG) IN .08 M H2SO4/Y/096 MORTALITY PCT
PER CENT
0.00
12.50
25.00
37.50
50.00
62.50
75.00
87.50
100.00
MG/KG
80.00
160.00
240.00
320.00
400.00

Table 21

DATA SUMMARY

$ZnSO_4$ - Rayon in .08 M H_2SO_4 in Water
via Air Cell at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	453	7.50	5.73	0.88
Solvent (pH control)	None	117	7.69	0.92	*
10.0	0.5	118	12.71	0.84	0.84
20.0	1.0	118	13.55	6.77	1.69
100.0	5.0	119	51.26	10.92	5.04
200.0	10.0	114	75.43	12.28	5.26
400.0	20.0	119	90.75[1]	16.80[2]	6.72[3]

[1] Difference from lowest test dose is highly significant

[2] Difference from test group showing least response is highly significant

[3] NS

[4] Regression of dose on mortality is highly significant

LC_{30} = 49 mgs./kg.
LC_{50} = 93 mgs./kg.
LC_{70} = 176 mgs./kg.
LC_{90} = 445 mgs./kg.

[5] Regression of dose on abnormal chicks is significant

*Appropriate data not calculated by computer and not used for statistical comparisons.

Table 22

DATA SUMMARY

Zn SO_4 - Rayon in Injected Control .0009 M H_2SO_4,
Compound Solvent .08 M H_2SO_4
vai Air Cell at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	453	7.50	5.73	0.88
Solvent (pH control)	None	109	3.67	0.95	*
0.78	0.039	109	7.33	9.17	0
1.56	0.078	110	16.36	7.27	1.81
3.12	0.156	69	49.27	18.84	2.89
3.56	0.178	40	15.00	20.00[2]	5.00[3]
6.25	0.312	108	82.40	11.11	1.85
12.50	0.625	109	92.66[1]	5.5	0.91

[1] Difference from lowest test dose is highly significant

[2] Difference from test group showing least response is significant

[3] NS

[4] Regression of dose on mortality is highly significant

LC_{30} = 2.6 mgs./kg.
LC_{50} = 3.9 mgs./kg.
LC_{70} = 5.8 mgs./kg.
LC_{90} = 10.2 mgs./kg.

[5] Slope is negative

*Appropriate data not calculated by computer and not used for statistical comparisons.

Table 23

DATA SUMMARY

Zn SO_4 - Rayon in .08 M H_2SO_4 in Water
via Yolk at 0 Hr.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	453	7.50	5.73	0.88
Solvent (pH control)	None	112	24.11	0	*
10.00	0.5	120	37.50	6.66	2.50
20.00	1.0	118	33.89	8.47	2.54
100.00	5.0	119	43.69	15.96	4.20
200.00	10.0	119	84.87	31.09[2]	13.44[3]
400.00	20.0	118	98.30[1]	5.93	4.23

[1] Difference from lowest test dose is highly significant

[2] Difference from test group showing least response is highly significant

[3] Same as 2.

[4] Regression of dose on mortality is highly significant

LC_{30} = 16 mgs./kg.
LC_{50} = 44 mgs./kg.
LC_{70} = 119 mgs./kg.
LC_{90} = 502 mgs./kg.

[5] NS

*Appropriate data not calculated by computer and not used for statistical comparisons.

Table 24

DATA SUMMARY

$ZnSO_4$ - Rayon in .08 M H_2SO_4 in Water via Yolk at 96 Hrs.

Dose of Compound Injected (mgs./kg.)	(mgs./egg)	Number of Eggs	Percent Mortality[4]	Percent Abnormal Chicks Hatched[5]	Percent H-S-V-L Abnormalities
Control	None	453	7.50	5.73	0.88
Solvent (pH control)	None	110	11.81	7.21	*
6.25	0.31	39	5.12	12.83	7.69
10.0	0.50	70	14.28	8.57	1.42
12.5	0.62	39	5.12	30.76	0
20.0	1.0	70	14.28	11.42	0
25.0	1.25	40	15.00	12.50	0
50.0	2.5	40	32.50	37.50	2.50
100.0	5.0	110	46.36	34.54	10.00
200.0	10.0	68	85.29	44.11[2]	19.11[3]
400.0	20.0	69	95.65[1]	23.18	15.94

1 Difference from lowest test dose is highly significant

2 Difference from test group showing least response is highly significant

3 Same as 2

4 Regression of dose on mortality is highly significant

LC_{30} = 54 mgs./kg.
LC_{50} = 92 mgs./kg.
LC_{70} = 156 mgs./kg.
LC_{90} = 334 mgs./kg.

5 Regression of dose on abnormal chicks is significant

* Abnormality data not calculated by computer and not used for statistical